Arnold Schwarzenegger
Governor

SEQUESTRATION OF CARBON DIOXIDE EMISSIONS THROUGH BIOCATALYTIC MINERALIZATION

PIER FINAL PROJECT REPORT

Prepared For:
California Energy Commission
Public Interest Energy Research Program

Prepared By:
Gas Technology Institute

July 2010
CEC-500-2009-088

Prepared By:
Gas Technology Institute
Project / Report Manager — Diane Saber / Xiangyang Zhu
1700 S. Mt. Prospect Rd.
Des Plaines, IL 60018
Contract No: 500-06-054

Prepared For:
Public Interest Energy Research (PIER)
California Energy Commission

Sarah Pittiglio
Contract Manager

Linda Spiegel
Program Area Lead
Environmental Area

Kenneth Koyama
Office Manager
Energy Systems Research Office

Thom Kelly, Ph.D.
Deputy Director
ENERGY RESEARCH & DEVELOPMENT DIVISION

Melissa Jones
Executive Director

Preface

The California Energy Commission's Public Interest Energy Research (PIER) Program supports public interest energy research and development that will help improve the quality of life in California by bringing environmentally safe, affordable, and reliable energy services and products to the marketplace.

The PIER Program conducts public interest research, development, and demonstration (RD&D) projects to benefit California.

The PIER program strives to conduct the most promising public interest energy research by partnering with RD&D entities, including individuals, businesses, utilities, and public or private research institutions.

PIER funding efforts are focused on the following RD&D program areas:

- Buildings End-Use Energy Efficiency
- Energy Innovations Small Grants
- Energy-Related Environmental Research
- Energy Systems Integration
- Environmentally Preferred Advanced Generation
- Industrial/Agricultural/Water End-Use Energy Efficiency
- Renewable Energy Technologies
- Transportation

Sequestration of Carbon Dioxide Emissions Through Biocatalytic Mineralization is the final report for the Sequestration of Carbon Dioxide Emissions Through Biocatalytic Mineralization project (Contract Number 500-06-054) conducted by Gas Technology Institute (GTI). The information from this project contributes to PIER's Energy-Related Environmental Research Program.

For more information about the PIER Program, please visit the Energy Commission's website at www.energy.ca.gov/pier or contact the Energy Commission at 916-654-4878.

Please cite this report as follows:

Aikens, John, Xiangyang Zhu, and Diane Saber. 2009. *Sequestration of Carbon Dioxide Emissions Through Biocatalytic Mineralization*, California Energy Commission, PIER Energy-Related Environmental Research Program. Publication Number: CEC-500-2009-088.

Table of Contents

Abstract v

Executive Summary 1

1.0 Introduction 5

2.0 Biomimetic Carbon Dioxide Sequestration: State of the Art 7

2.1. Biomimetic CO_2 Sequestration: Biocatalysis 7

2.2. Biocatalyst Selection: Performance Criteria 10

2.3. Biocatalyst Immobilization: A Key Feature of Biomimetic Sequestration 12

2.4. Bioreactor Design 14

2.5. Bubble Chambers 16

2.6. Multiphase Contactors 17

2.7. Membrane Partition Reactors 18

3.0 Cloning, Expression, and Characterization of Carbonic Anhydrase 21

3.1. Cloning Carbonic Anhydrase 21

3.2. Choice of Carbonic Anhydrase (CA) Enzyme 21

3.3. Production and Isolation of Carbonic Anhydrase 23

3.4. Immobilization of Carbonic Anhydrase 28

4.0 Bioreactor Design and Testing 31

4.1. Bioreactor Construction 31

4.2. Bioreactor Testing 33

5.0 Conclusions and Recommendations 37

5.1. Conclusions 37

5.2. Recommendations 37

5.3. Benefits to California 38

6.0 References 41

7.0 Glossary 45

List of Figures

Figure 1. X-ray crystal structure of α-carbonic anhydrase 9

Figure 2. General process flow diagram of a gas phase to solid conversion and sequestration of CO_2. 16

Figure 3. Multiphase contactor reactor. 18

Figure 4. Membrane partition reactor. 19

Figure 5. Sequence of the alpha-class CA from *Streptococcus thermophilus* LMG 18311, StCA-His_6 22

Figure 6. Plasmid pLybCD5 for the expression of StCA-His_6 in *E. coli*. 23

Figure 7. Expression and purification of carbonic anhydrase. 26

Figure 8. Purification results for CA isolation. 26

Figure 9. The assay of purified cloned carbonic anhydrase. 28

Figure 10. Activity retention of immobilized carbonic anhydrase. 29

Figure 11. Conceptual bioreactor design. 32

Figure 12. Photograph of prototype laboratory sequestration bioreactor 33

Figure 13. Plot of bioreactor pH shift. 35

List of Equations

Equation 1. Key chemical equations associated with the hydration of CO_2 7

List of Tables

Table 1. Culture media composition for growing recombinant *E. coli* hosts bearing the plasmid coding for carbonic anhydrase 25

Abstract

Carbon dioxide levels in the atmosphere have steadily increased throughout human history; however, the most dramatic changes have occurred since the dawn of the Industrial Revolution. Continued unmitigated elevations in this greenhouse gas are believed to pose a threat to global ecosystems, a threat that compels the development of methods to reduce emissions from human activity. Nature has evolved to capture and use carbon dioxide and represents one of the primary mechanisms for sequestering this gas from the environment. Using microbial DNA cloning techniques common in industrial protein production and the chemical process of hydration of carbon dioxide, the project demonstrates the feasibility of an innovative approach to carbon dioxide sequestration that mimics already existing biological functions. The biochemical approach to carbon dioxide removal effectively and directly converts gaseous material passing through a reaction chamber to simple salts that are continuously removed from the system. The prototype design is based on materials that can be readily scaled and fabricated to be adapted to industrial-scale point sources of carbon dioxide emissions. This technology has the potential to assist California industry in meeting greenhouse gas emissions reduction requirements in a cost-effective and environmentally friendly manner.

Keywords: Carbon dioxide, carbonic anhydrase, protein immobilization, gas phase biocatalysis, genetic engineering

Executive Summary

Introduction

Electricity production through fossil fuels combustion has resulted in a dramatic increase in the levels of the greenhouse gas carbon dioxide. In spite of intense research in alternative energy development, the combustion of carbon fuels will be the primary source of energy for the foreseeable future. Effective management of carbon dioxide waste streams is critical to reduce global warming and protect global ecosystems. One of the most significant resources for carbon dioxide removal can be found in biological systems. The growth of photosynthetic organisms such as green plants, algae, and cyanobacteria (blue-green algae) is responsible for a significant portion of moving carbon dioxide through the global carbon cycle[1]. Biological carbon dioxide sequestration represents a powerful process that can serve as a permanent, safe, and environmentally friendly means to sequester carbon dioxide from point sources of emission.

Purpose

This project serves the California Energy Commission's Public Interest Energy Research Program goal of improving the environment, public health, and safety of California's electricity by assessing the feasibility of an enzyme-based process for reducing or eliminating carbon dioxide emissions.

Project Objectives

The project objectives are to 1) evaluate the state of the art for enzyme-based sequestration technologies; 2) produce the most appropriate enzyme for the sequestration process and immobilize it within a solid matrix to promote sequestration of carbon dioxide; and 3) construct a laboratory-scale bioreactor using enzyme-based carbon dioxide sequestration and perform preliminary reactor trials and analysis.

Project Outcomes

The state-of the-art review demonstrated that the principle of enzyme-based biosequestration is a viable green alternative for removing carbon dioxide from the environment. The results from the preliminary bioreactor trials established that it is feasible for a multiphase bioreactor[2] to successfully sequester carbon dioxide to bicarbonate ion using enzyme carbonic anhydrase[3]. Although this technology has not been tested on a large scale, the results demonstrate that scaling the project up for on-site industrial use is feasible.

---

[1] The biogeochemical cycle in which carbon is exchanged among Earth's biosphere (broadly, all ecosystems), pedosphere (soils), geosphere, hydrosphere, and atmosphere.

[2] A vessel of complex design in which chemical processes are carried out using biological organisms or biochemically active substances (such as enzymes) from biological organisms.

[3] A family of enzymes that catalyze the rapid conversion of carbon dioxide to bicarbonate and protons.

Conclusions

It is expected that as biosequestration processes mature from the laboratory and pilot plant stage and into demonstration scales, the practical implementation of these technologies will become a reality. An economic analysis of carbon dioxide sequestration done with biological catalytic agents[4], such as the carbonic anhydrase enzyme, has yet to be developed and likely will depend on the collection of data from larger-scale demonstration of various reactors installed in real applications. In spite of limited application knowledge, current understanding of biocatalytic sequestration suggests that this approach to managing carbon dioxide waste streams warrants further evaluation and development.

Recommendations

Major unmet challenges still remain with biocatalytic sequestration of carbon dioxide. The two most important considerations involve the type of enzyme used and the secondary processing of carbonate products. The former issue will require continued search for enzymes that have desirable characteristics, such as heat tolerance, tolerance of high salt concentrations, and low production costs. The concern over secondary processing involves identification of methods and materials that will enable efficient capture and recovery of the carbonate product from bioreactor solutions. Two ongoing challenges to the large-scale implementation of the technology include identifying the source of the counter ions needed to facilitate carbonate precipitation from bioreactor solutions and the subsequent removal and storage of potentially large volumes of that solid carbonate precipitate. In spite of the remaining hurdles associated with biocatalytic carbon dioxide sequestration, there is enough evidence to suggest that these processes be considered seriously as complementary approaches to carbon dioxide removal from the environment.

Benefits to California

California has emerged as a leader in developing environmental policy and programs in the United States. Its large population, unique geographic features, and diversity of natural resources create challenges for the state to balance resource use, commerce, recreation, and responsible stewardship of lands. Air, land, and water pollution is a persistent consequence of large population densities and robust economic activity and requires ambitious intervention to avoid profound damage to California's environment.

Human activity has been cited as a primary mechanism for the dramatic increase in atmospheric concentrations of carbon dioxide. California has recognized the role of responsible environmental stewardship and is cognizant of the need for introducing innovative means to combat pollution and specifically carbon dioxide emissions. The use of biosequestration technologies to combat progressive increases in carbon dioxide emissions represents a green alternative that can serve as a permanent, safe, and environmentally friendly means to

[4] A process known also as biocatalytic sequestration.

sequester carbon dioxide from point sources of emission. As with other pollution remediation issues, government intervention is required to compel both commercial interests and the residents to recognize the overall benefits of environmental responsibility. Actions already underway within California provide means that will allow sequestration and carbon dioxide emissions management to become reality. The efforts of this project provide the opportunity to add a practical tool to enable point-source carbon dioxide emissions sites to economically implement carbon dioxide sequestration programs.

1.0 Introduction

Since the Industrial Revolution, the production of energy through the combustion of fossil fuels to spur human activity has resulted in a dramatic increase in atmospheric greenhouse gas carbon dioxide (CO_2) (Schmalensee et al. 1998). The spread of industrial resources, distribution of wealth, and increase in the quality of life throughout the world coupled with continuing growth in the human population supports the forecast of continued increases in atmospheric CO_2 levels (EIA). While the scientific community has successfully educated the population about the potential consequences of uncontrolled greenhouse gas emissions, the challenges to stem emission increases are almost overwhelming. In spite of intense research in alternative energy development, the fact remains that combustion of carbon fuels will be the primary source of energy for the foreseeable future. Given the worldwide demands for energy, there it is critical to address managing resultant CO_2 waste streams.

While a number of different possibilities have been considered, each have limits and challenges for practical, economic implementation. Interestingly, little research has been applied to biomimetic CO_2 sequestration technologies, although one of the most significant resources for CO_2 removal can be found in biological systems (Lal 2008). The growth of photosynthetic organisms such as green plants, algae and cyanobacteria is responsible for a significant percentage of planetary carbon cycling. In spite of the profound amount of CO_2 that is converted to biomass by photosynthetic organisms, the levels of CO_2 in the environment continue to rise. The challenge with biological CO_2 sequestration is that the overall natural rate of CO_2 uptake is relatively slow and can be overwhelmed by continued increases in energy consumption. The rate of CO_2 emissions from human activity has outpaced the capacity of biological or natural sequestration -- nature needs assistance to clean up CO_2 generated by human activity. What is also clear is that while biological sequestration in the environment may have approached saturation, the fundamental mechanism of biological sequestration clearly is a powerful process for managing CO_2 emissions. If the key elements for CO_2 sequestration used by biological systems can be understood, valuable biomimetic processes can be developed as a means to reduce atmospheric CO_2.

The enzyme carbonic anhydrase is the focus of this investigation, as it is uniquely suited to large-scale CO_2 sequestration. Carbonic anhydrase catalyzes the hydration of CO_2 at rates of more than 1 million molecules of CO_2 per second. This means that less than 1 kilogram of carbonic anhydrase will be necessary to sequester the approximately 290 tons of CO_2 produced per hour from a typical 300-MW coal-fired power plant. The enzyme is ubiquitous in nature in bacteria, plants, and humans, and plays an important role in physiology. Further, calcium or magnesium carbonate, the solid product of enzyme-based CO_2 sequestration, is a safe, stable material that can be land-disposed or used in diverse applications.

This project meets the PIER Goal of Improving the Environment, Public Health, and Safety of California's Electricity by assessing the feasibility of an enzyme-based process for reducing or eliminating CO_2 emissions. The project objectives are to 1) evaluate the state of the art for enzyme-based sequestration technologies, 2) produce the selected carbonic anhydrase enzyme

and immobilize the enzyme to a solid matrix for CO_2 sequestration, and 3) construct a laboratory-scale bioreactor utilizing enzyme-based CO_2 sequestration and perform preliminary reactor trials and analysis.

2.0 Biomimetic Carbon Dioxide Sequestration: State of the Art

2.1. Biomimetic CO_2 Sequestration: Biocatalysis

The key to biological sequestration is the hydration of carbon dioxide in water to form the carbonate anion (Al-Rawajfeh 2004). This is an equilibrium process that occurs spontaneously and the chemistry of the uncatalyzed reaction is shown in Equation 1. Hydration of CO_2 begins with the partitioning and dissolution of gaseous CO_2 into an aqueous environment. This dispersion of gas into liquid phase is a complex process involving mass transfer, gas partial pressure, activity of the aqueous phase and temperature. Because the solubility of CO_2 in water is approximately 30 millimolar, the solubility of the gas in aqueous environments generally follows Henry's Law (Gerrard 1976). The temperature and pH of the aqueous solution can dramatically influence the behavior of CO_2 in the solution. In pure water the CO_2 generally undergoes dispersion and solubilization according to equation (1). In a slow reaction, water can then react with CO_2 according to equation (2) to ionize CO_2 to create carbonic acid. This reaction is slow because water is a relatively poor nucleophile bearing no net charge. If no base is present in the solution, CO_2 reaches equilibrium governed by equations (1) and (2). The chemistry described in Equations (3) and (4) become the dominant chemistry between pH's 6 and 9. These reactions are very rapid and depend on the concentration of the strong nucleophile hydroxide ion. The observed rate of the reaction therefore is pH dependent. Under alkaline aqueous conditions, CO_2 reacts with hydroxide ions very rapidly according to equation (5). Thus, the complex chemistry of CO_2 in aqueous environments creates significant challenges for using liquid phases for sequestration applications.

$$CO_2(g) \rightleftharpoons CO_2(aq) \quad (1)$$

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \quad (2)$$

$$H_2CO_3 + {}^-OH \rightleftharpoons HCO_3^- + H_2O \quad (3)$$

$$HCO_3^- + {}^-OH \rightleftharpoons CO_3^{=} + H_2O \quad (4)$$

$$CO_2(aq) + {}^-OH \rightleftharpoons HCO_3^- \quad (5)$$

Equation 1. Key chemical equations associated with the hydration of CO_2

According to the chemical reactions described, the uncatalyzed formation of carbonate from CO_2, which is critical to the removal of CO_2 from the gas phase and therefore exhaust waste streams, only occurs at the high reaction rates required under basic conditions. This makes one of the major challenges for the sequestration of CO_2 managing the pH of the hydration environment. Since the base in solution is consumed upon addition of hydroxide to the CO_2, the hydration of CO_2 effectively acidifies the liquid phase as the gas is ionized. Thus the effective

capture of significant amounts of CO_2 requires highly basic saline solution conditions that pose operational hazards, as well as challenges associated with damage to the reactors, pipes and machinery that contact the solution (Kroschwitz and Howe-Grant 1996).

What is needed is a means to eliminate the chemical requirement for harshly basic conditions to efficiently convert CO_2 to carbonate. To this end, chemical catalysis using polymer supported amines have been investigated and demonstrated to effectively trap CO_2. Cost and scalability of these systems, however, continues to limit large scale implementation of these systems (Pinola et al. 1993; Barth et al. 1984; Sartori et al.; Yoshida et al.; Fujii et al.; Suzuki et al.). Another alternative to chemical catalysis, though, is the use of a biomimetic approach to CO_2 hydration. The key element in understanding biological sequestration is the identification of the mechanism by which biological systems manage and sequester atmospheric CO_2. While the overall process of biological sequestration is complex and highly controlled, the key component of the conversion of CO_2 to carbonate comes down to the chemistry of a single enzyme catalyst (Smith and Ferry 2000; Lindskog 1983).

The enzyme carbonic anhydrase is responsible for converting CO_2 from the gas phase to carbonate ions that are soluble in the liquid phase. Carbonic anhydrase, which is one of the most ubiquitous and important proteins known in biology, is really a single name for a group of proteins that vary in structure and function (depending on the organism) but share the common chemistry of catalyzing the hydration of CO_2 to form carbonate. The enzyme, regardless of structure, is generally believed to be highly adapted to the ionization of CO_2 in the sense that the reaction rates are almost diffusion limited (Khalifah and Silverman 1991). Their observed rate is so fast and efficient that as many as 1 million CO_2 molecules can be converted to carbonate every second for each carbonic anhydrase molecule. This phenomenon is more remarkable given that the enzyme works in neutral to slightly acidic environments, which under normal solution conditions would preclude any significant hydration of CO_2. Viewed in chemical terms, the enzyme as shown in Figure 1 functions by activating hydroxyl ions formed through ligation to a zinc atom bound within the enzyme (Silverman and Vincent 1983). The enzyme further acts to hold CO_2 in the active site close to the activated hydroxyl group, while at the same time preventing water from gaining access to the hydroxyl group. The enzyme, thus, creates a microenvironment of extreme basicity, which allows for the rapid reaction of equation (5) to occur without altering the pH of the bulk solution.

Carbonic anhydrases have been extensively researched and a great deal is understood about their functional properties, particularly as they relate to their role in biological systems. The biological function of carbonic anhydrase is generally the maintenance of pH in biological solutions. pH plays a major role in metabolism, respiration and consequently disease. In this context, carbonic anhydrase has been viewed as a primary drug target for a wide range of diseases, from glaucoma to bacterial infections to cancer (Pastorekova et al. 2004). In photosynthetic organisms, carbonic anhydrase also functions to sequester CO_2 to increase the efficiency of carbon fixation (Forsman 2000). It stands to reason that this body of knowledge can be recruited for use in developing a non-biological application for the enzyme. In principle, a carbonic anhydrase, which has high kinetic rates and can be isolated from a wide variety of

organisms that thrive in almost any environment, could be developed for the sequestration of CO_2 in gas streams.

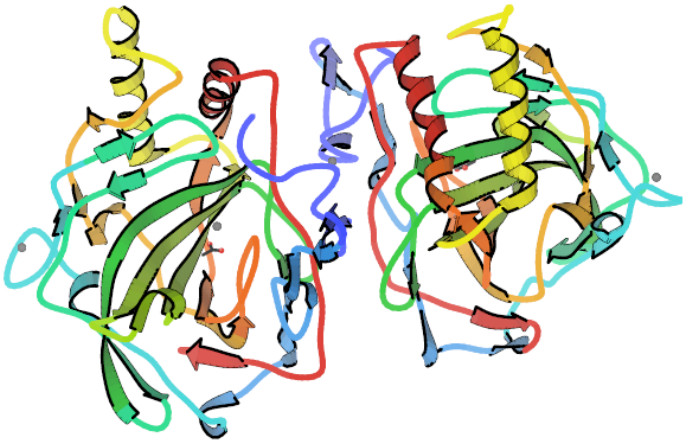

Figure 1. X-ray crystal structure of α-carbonic anhydrase (Premkumar et al. 2005). Image rendered depicts the dimeric structure of the holoenzyme. The active zinc atom is protected from the bulk solvent by location within the active site of the enzyme. The enzyme shown is from a halotolerant bacterium. However, it has very high homology to other α-CA enzymes, including the human type II α-CA.

Source: Authors

From the standpoint of CO_2 sequestration, carbonic anhydrase plays the role of a chemical catalyst that is responsible for accelerating the hydration of CO_2 to form carbonate. Unlike conventional chemical catalysts, however, carbonic anhydrase is a complex macromolecular polymer of amino acids that is folded to form a specific structure suited for its catalytic function. In addition, this structure is flexible and capable of "breathing" in response to the reaction cycle, which is believed to provide some of the energy associated with catalysis. This mobility can lead, under certain conditions, to complete unfolding of the enzyme, which typically results in the irreversible loss of activity.

Chemists and engineers have historically viewed biological materials, including enzymes, as sensitive, fragile and fickle systems that are not suited for the demanding and often extreme environments found in industrial applications (Faber 1992). This perspective is largely a result of misunderstanding the operating criteria for enzymes. While there is no doubt that there are limits to the environment that enzymes can operate within, the fact that these systems function at ambient temperatures in aqueous solutions provides a unique opportunity to create efficient processes that do not require heat, pressure or extreme conditions, all of which add costs to processes. If an enzyme based process can be developed, there is a strong likelihood that the overall costs of CO_2 sequestration could achieve key thresholds of economic viability.

2.2. Biocatalyst Selection: Performance Criteria

There are a number of technical issues that must be addressed in selecting a carbonic anhydrase for use in industrial carbonic anhydrase applications. Almost every living organism has at least one type of carbonic anhydrase, which provides a large pool of enzymes possessing a wide variety of properties that can be selected, depending on operational considerations. While this initially seems to be a daunting task, there are a number of performance criteria that narrow the likely candidates to a manageable subset. Carbonic anhydrases are classified into 4 basic categories, based on the sequence of the polypeptide and the overall assembly of the polymeric chains (Tripp et al. 2001). Most of the carbonic anhydrases known fall into classes β, γ, and ε, which are enzymes that are assembled into complex structures such as trimers, tetramers, and octamers. These complex structures pose a challenge for their production and processing in active form.

The α class enzyme by contrast is a small polypeptide with a molecular mass of approximately 25-30 kilodaltons that form a simpler dimeric quaternary structure that binds a single zinc ion at each active site. This class of enzyme is by far the best studied of the carbonic anhydrases, having been first identified and isolated from human red blood cells in the 1930's (Meldrum and Roughton 1998). To date, the human enzyme has been cloned, genetically engineered and manipulated to reveal critical mechanistic functions and is currently the enzyme of choice for CO_2 sequestration studies (Daigle et al. 2006). From the perspective of CO_2 sequestration, only the α-class, recombinant type II human carbonic anhydrase has been studied and is being entered into pilot plant level sequestration trials.

While the enzyme is a very rapid catalyst and has been demonstrated to function effectively in laboratory scale experiments, challenges remain with the use and production of this enzyme that compel the search and development of alternative enzyme activities. Most notable among the concerns are the poor expression levels of the enzyme, its relative vulnerability and intolerance to saline environments and inhibitor ions, and its potential for unfolding in response to temperature and pH variations. In an effort to circumvent stability issues, genetically engineered recombinant human carbonic anhydrase has been studied and found to have modestly improved stability without compromising catalytic activity (Daigle et al. 2006). In spite of the improved stability, expression and production of the enzyme in a scalable cost-effective system has remained elusive.

Very recently, an α-type carbonic anhydrase has been considered for use which is isolated from the thermophilic organism *Methanosarcina thermophila*. This enzyme has not been studied extensively and because of its complex structure, it is unclear whether a cost effective production process will or can be developed (Trachtenberg 2008; Alber and Ferry 1994). An alternative enzyme candidate will likely come from a halophilic and thermophilic bacterium that possesses an α-class carbonic anhydrase. This enzyme will display both tolerance to high saline environments and be resistant to inactivation from inhibitors or environmental conditions. Since it is from a bacterial source, production in recombinant form in bacterial hosts is potentially less problematic. A robust carbonic anhydrase that can be produced in high yield will favorably impact production and operational costs by affording more enzymes per weight

biomass, which will enable more efficient immobilization and improve bioreactor active lifetimes.

The issue of enzyme production has become a significant concern and major limitation for application of α-class, recombinant type II human carbonic anhydrase and is considered a key performance criterion for enzyme selection. This requirement is critical for industrial application, since it is expected that large quantities of enzyme will be required for sequestration. The common perspective on economic production of biocatalysts involves the preparation of recombinant enzymes produced in bacterial hosts. Bacteria are generally utilized because they can be grown quickly, for low cost and are well characterized as production systems for enzymes. The key to working with bacterial systems as biocatalyst factories is to develop an optimized protein expression process. Several important considerations are necessary when designing the system, such as DNA sequence compatibility, enzyme stability, enzyme toxicity and susceptibility of the produced enzyme to proteolysis (Arnold and Volkov 1999). Once again α-class enzymes, owing to their small size and relatively noncomplex structure, are good candidates for recombinant production in bacteria. A number of protein expression systems exist for the human type II enzyme (Forsman et al. 1988). Many of these bacterial production systems have been developed for academic research purposes and have not been optimized for large scale production. While the human type II carbonic anhydrase is the current state of the art enzyme, the recombinant human enzyme suffers from low expression yields, which is not considered to be economically viable on an industrial scale. There are a number of potential reasons for the relatively low yield of isolated enzyme, but it is likely that a contributing factor is the fact that the DNA sequence of the enzyme from humans is not entirely compatible with expression machinery of bacterial hosts.

A third consideration for enzyme performance is tolerance to the intended operational environmental conditions. The typical application for carbon sequestration would involve a device that contained the enzyme positioned in order to allow a stream of flue gas from a combustion source to pass over the enzyme. The source of flue gas can be envisioned as a stream from a power plant operating with coal, natural gas or oil. The CO_2 levels in these gas streams have historically presented challenges for sequestration, since the concentration of carbon is quite dilute, typically on the order of 8-12 volume percent (Department of Energy 1991). In addition, the total flow of gas from a combustion source such as a power plant can be quite high. Other factors present in a typical exhaust stream are also expected to have a significant potential impact on a biological sequestration strategy. Carbon dioxide sequestration in flue gas applications is expected to involve exposing the enzyme to elevated temperatures. For all practical purposes there is an upper limit of temperature tolerance for enzymes of between 20ºC and 80ºC, which means that gas streams will need cooling prior to enzyme exposure (Spelman et al. 2001). Other by-products of the combustion process such as phosphates, nitrogen and sulfur oxides, in addition to ash and other minerals, may cause inhibition of enzyme function and need to be assessed. If necessary, these materials would also need to be processed prior to the gas stream being exposed to the enzyme reactor.

While the issue of temperature will require an engineering solution prior to enzyme exposure to the exhaust stream, the enzyme appears to be quite tolerant to the presence of combustion by-products (Electric Power Research Institute 1999). Several studies of model flue gas streams have indicated that the recombinant human carbonic anhydrase appears to tolerate many of the components of flue gases, with the exception of monovalent anions such as chloride (Maren et al. 1976). The enzyme does not appear to be well suited for highly saline environments and is not very tolerant to elevated temperatures. Genetically engineered variants of the human enzyme have been produced that resist modest increases in temperature, but these improvements may still not be sufficient for application. An ideal enzyme would likely come from a halophilic and thermophilic bacterium. There are relatively few of these bacteria available in pure culture, fewer that have known DNA sequences for manipulation and almost none known to possess an α-class carbonic anhydrase. To date, only one enzyme has been identified in our laboratory that matches this description, which creates an opportunity to expand the discovery and development process.

2.3. Biocatalyst Immobilization: A Key Feature of Biomimetic Sequestration

One of the most important considerations for a biocatalytic reaction system is the challenge of delivering CO_2 to the enzyme while removing carbonate products. The historical viewpoint of enzymes requiring an aqueous phase for function and stability has dominated the application of enzyme processes. Over the last 10 years however enzyme technology development has demonstrated that enzyme function is not limited to aqueous environments. Recently, research has shown that enzymes can function extremely well even in the gas phase (Letisse et al. 2003). These revelations have prompted a broadened perspective of enzyme process development and are expected to contribute new breakthroughs in bioprocess development, as it pertains to CO_2 sequestration.

Research focused on biocatalytic processes for CO_2 sequestration has identified the need to immobilize the enzyme to a surface. The reasoning behind this approach is that an interfacial gas and liquid boundary is generally required to effectively transfer gaseous CO_2 into an ionic form. Immobilization serves the dual purpose of holding the enzyme in the appropriate location to affect catalysis and prevent the enzyme from being washed out of the reactor. In addition, it has been shown that immobilized enzymes are more stable and resist unfolding when in the immobilized state compared to those that remain free in solution (Martinek and Mozhaev 1985). This latter observation enables an enzyme that ordinarily would be vulnerable to environmental inactivation to resist damage and function for extended periods of time. There have been a number of strategies used to immobilize carbonic anhydrase to solid supports. Three basic strategies have been explored: physical entrapment, crosslinking, and high affinity binding.

2.3.1. Matrix Immobilization

Physical entrapment involves formulating the enzyme in solution with specific trapping agents such as alginate or other polymeric resins (Simseket al. 1999). The trapping agents are then activated, which causes them to solidify and hold the enzyme within a solid matrix. The final

solid is a gel-like material that allows water, ions and gases to permeate and exchange between the outer and inner surfaces. The advantage to this approach is that the enzyme is trapped in its native state, allowing it the necessary freedom to "breathe" for catalysis, without providing enough space for the enzyme to fully unfold. In this manner, the immobilization matrix limits inactivation by unfolding by creating a semi-rigid scaffold around the enzyme. The difficulty with these systems is that they need to be kept from drying to maintain function and, depending on the thickness of the material, the rate of catalysis can be limited by permeation. In addition, the process for producing entrapped enzymes can be labor intensive and difficult to control, which raises the potential of poor reproducibility. The materials used as trapping agents are also single use and costly, which creates challenges of affordability on large scales.

2.3.2. Surface Crosslinking

Covalent crosslinking is a popular and common practice for immobilizing enzymes (Boller et al. 2002). The state of the art processes being developed for CO_2 sequestration employs some form of crosslinking of the carbonic anhydrase to a surface. Two related methods are currently being used, epoxy-activated and aldehyde-activated covalent crosslinking. In both instances, the immobilization surface (beads or membranes) are treated with chemical reagents that install the appropriate chemical functionality. The enzyme is then mixed and reacted with the functional groups on the immobilization surface, which generally react with amine groups on the solvent exposed outer portions of the enzyme. The chemical reaction between the enzyme amine groups and the crosslinking agent attached to the immobilization surface forms a permanent bond that fixes the enzyme to the surface material. The enzyme is then ready for use in a bioreactor for reaction with CO_2. While this approach is a relatively simple scalable process, there are a number of issues that create concern with respect to large scale application.

The two most notable are the relatively indiscriminant reactivity of the crosslinking groups and the requirement for a purified carbonic anhydrase enzyme. The former chemical reactivity issue is important because the functional groups (epoxy and aldehyde) are highly reactive toward enzymes. It is common that either multiple crosslinks are formed or chemical reactions occur in or near the active site. If the former multiple reactivity occurs, the enzyme can be unfolded and inactivated by the surface or it may be bound in such an orientation as to block access to the catalytic active site. If the latter active site reaction occurs, the site can be blocked or key residues involved in catalysis can be altered by the cross linking, which prevents the catalytic cycle from operating. In either case, the amount of active enzyme bound to the surface can be significantly lower than expected, which makes the bioreactor less productive.

The second important issue is the need to purify the enzyme prior to immobilization due to limited surface availability. This concern has been a major challenge for bioprocessing applications for a long time. The two concerns that afford significant consequences are the processing costs of purifying the enzyme prior to immobilization and the significant loss in productivity from binding undesired protein to the surface. The former concern of cost can be significant, since enzyme purification can contribute the majority of costs associated with using purified enzymes. The costs are generally high enough to prevent biocatalysis from being considered as a reasonable alternative for many industrial applications. The latter concern of

crosslinking undesired enzyme is significant because in many cases most of the crosslinking agent and thus immobilization surface is tied up with protein that is unproductive. This becomes a major challenge as in the case of human carbonic anhydrase, since the overall expression of the recombinant enzyme in bacterial hosts is unacceptably low.

2.3.3. Physicochemical Binding

The third general method for immobilizing enzyme is through physical adsorption to a surface or binding to a high affinity linker (Montero et al. 1993). Physical surface binding is a very inexpensive and nonselective process by which a material such as nitrocellulose or certain clays are mixed with enzymes. The enzymes are attracted to the surface and held in place by electrostatic interactions. This approach involves relatively weak binding and generally has limited usefulness in large scale applications because the biocatalysts leach from the surface. An alternative process involves a high affinity linker binding technology that selectively attaches to and binds the desired enzyme to a surface (Porath et al. 1975). This process generally requires that the enzyme be genetically engineered to install a specific element that promotes selective and tight binding to an immobilization surface modified to possess a linked binding functionality. Human carbonic anhydrase has been engineered to be linked with affinity immobilization, however, more research and process optimization is needed to develop this method. The issues currently outstanding are that there are limited surface chemistries available and the process is not currently set up to permanently bind the enzyme. Depending on conditions, the enzyme can wash off the surface, which is problematic and clearly undesirable.

2.4. Bioreactor Design

Currently, existing systems require dispersal of a gas phase into a liquid system that enables the enzyme catalyst to function. Such designs, while functional are not easily scaled or practical in commercial application. The new design is needed that will affix the enzyme catalyst in such a manner as to enable direct interaction with gas phase thereby bypassing the partitioning CO_2 gas into a liquid phase prior to hydration (Benevides et al. 2003). In order to accomplish such "gas phase biocatalysis," the enzyme needs to be immobilized to a surface that can tolerate exposure to gas phase but at the same time enable liquid percolation to enable product carbonate transport. Woven silica fabrics are an example of materials that have been used as filtration media in air/particle separations and can be engineered easily for use as a "membrane" support for enzymes.

The "reactor" itself is designed to separate liquid from gas phase using the silica fabric "membrane" thereby enabling starting materials (CO_2) and products (carbonate salts) to be separated. Unlike other membrane or contactor designs the current project reactor employs the separation surface as a permeable interface that holds the enzyme in the appropriate position to maximize interaction with gas phase substrate while allowing water soluble product salts to be carried away by equilibrium controlled diffusion. Placing the enzyme on the buffer wetted gas phase surface minimizes partitioning phenomena of CO_2 through a liquid intermediary layer prior to binding in the catalytic site of the enzyme. The product upon diffusing into the liquid phase is transported into bulk solvent and carried to a bed of material that removes the

carbonate salt by ion exchange/precipitation. One of the most important considerations for a biocatalytic reaction system is the challenge of delivering CO_2 to the enzyme while removing carbonate products.

The design of bioreactors has focused on partitioning gaseous CO_2 into the liquid phase for carbonic anhydrase catalysis (Figure 2). The principle hurdle to aqueous based systems is the limited solubility of CO_2 in water (Suchdeo and Schultz 1974). The saturation point of CO_2 is 30 millimolar, which means that most of the CO_2 gas bubbled into water passes through without actually being dispersed. Large volumes of aqueous phase are required to dissolve significant quantities of CO_2. Each ton of coal produces approximately 2.3 tons of CO_2 which corresponds to roughly 15 tons of water needed to solubilize the CO_2 produced. In a typical coal fired power plant producing 300 MW of electricity, about 100 tons of coal is consumed per hour which would require 1500 tons of water to dissolve the CO_2 in the flue gas stream (Yegulalp et al. 2001). Clearly the limited solubility of CO_2 in water creates significant challenges of mass transport simply to deliver CO_2 to the carbonic anhydrase enzyme. This means that even though the enzyme is converting CO_2 in solution to carbonate nearly instantaneously, the reactive surface area of enzyme relative to the volume of aqueous environment creates an overall conversion process that is limited by mass transfer rate of CO_2 through the media, which is an inefficient process. A bioreactor design therefore needs to consider how to minimize the partitioning and dissolution of CO_2 into an aqueous medium prior to reaction with carbonic anhydrase. Preferentially, the reactor theoretically should have the gas stream in direct contact with the enzyme so as to avoid the liquid phase transfer altogether.

Delivering CO_2 to the enzyme for reaction is only part of the challenge. The carbonate product is an ionic material that is a solid, which is very soluble in an aqueous medium. The best way to transport and work with carbonate is as a solubilized salt in the liquid state. So while the CO_2 is best managed in a gaseous state, the product carbonate is best suited in the liquid state. It stands to reason that the ideal bioreactor would minimize the partitioning of CO_2 into a liquid phase but provide ready access to a liquid phase for carbonate product removal. A membrane-separated bioreactor is expected to meet these criteria as the enzyme is presented to the gas phase for CO_2 access, while the liquid phase permeates the membrane and enables the carbonate product to be removed. A secondary consideration for the process would be to maintain the pH of the liquid phase as the concentration of the acidic carbonate product increases. This is a significant challenge since buffer salts are generally viewed as the practical means to accomplish pH balance. The buffering of the liquid phase is dependent on the strength of the buffer solution and the concentration of carbonate in the system. Too low of a buffer concentration and the amount of carbonate retained before the pH of the solution becomes acidic is low. The concentration of carbonate in the liquid phase should be as high as practical in order to economically and efficiently transport and remove carbonate from the system. This is expected to require that the buffer system used will create a highly saline environment, which could negatively impact the catalytic activity of the enzyme. An alternative approach has been demonstrated in which a partitioning membrane reactor is used to convert CO_2 in a dilute gas stream into carbonate dissolved in a buffered aqueous system, and then on a second surface the

carbonate is re-converted back to CO_2 for release into a second gas stream (Cowan et al. 2003). The purpose of the process is to purify and concentrate the CO_2 so that it can be transported and stored. The concern of this approach is that while this appears to show economic promise for concentrating CO_2 compared to conventional technologies, the CO_2 is ultimately not removed from the environment but rather stored.

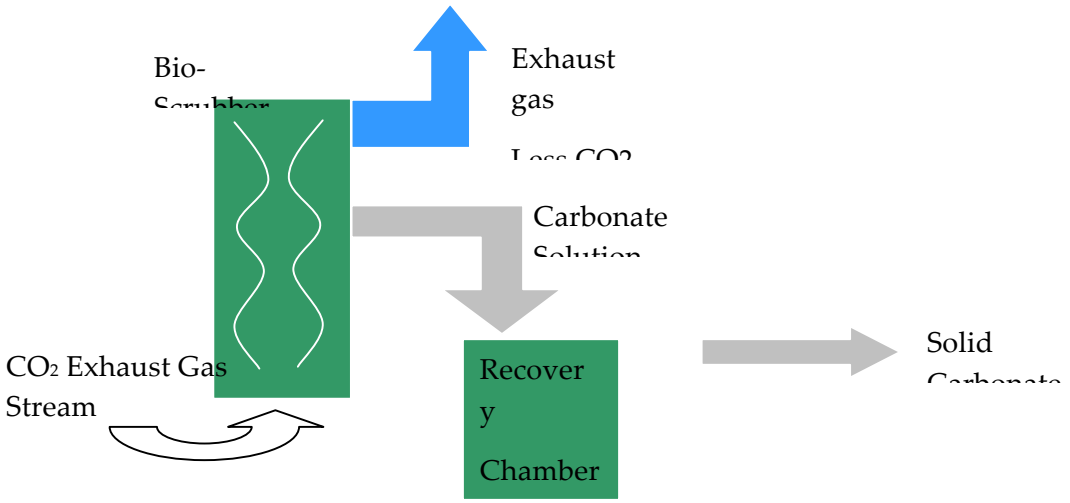

Figure 2. General process flow diagram of a gas phase to solid conversion and sequestration of CO_2. Depending on the nature of the bioscrubber, either multiphase contactor or membrane permeation type the waste gas stream enters the scrubber and is converted to carbonate. The effluent gas is then released to the environment depleted of CO_2. The ionized carbonate is then removed from the scrubber and transported to a recovery chamber where the carbonate is precipitated in a salt form using calcium or magnesium. The recovery chamber may be a countercurrent liquid reactor using calcium or magnesium brines or a heterogeneous bed of calcium or magnesium enriched clays. The transport solution once treated in the recovery chamber is then able to be recycled back to the bioscrubber. The solid carbonate is then removed for disposal.

Source: Authors

There are three bioreactor designs that have been described in the scientific and patent literature for biocatalytic CO_2 sequestration. These reactors can be defined as bubble chambers, multiphase contactors and membrane partition exchange systems (Treybal 1980). The goal of each reactor is the same in that they are designed to deliver CO_2 into a solution that enables the catalyst to carry out the hydration reaction. The carbonate product is then removed from the liquid phase in a secondary process, which allows the cycle to continue. Each of these designs provides different advantages and challenges for use in CO_2 sequestration. It is clear that while these bioreactors are being developed and scaled into larger demonstrations, considerably more effort will be required before an economical application-ready device will be available.

2.5. Bubble Chambers

The first and most straightforward is a chamber that is filled with buffered aqueous solution (Ramachandran and Chaudhari 1983). CO_2 is introduced by a glass frit located at the bottom of

the reactor and the enzyme is entrained in either an alginate or other immobilization matrix. The key aspect of the immobilized enzyme is that it is located within the immobilization matrix, which means that most of the enzyme is located on the inner surface of the immobilization material. The CO2 is converted to carbonate as the bubbles dissolve and partition the gas into solution, which is contacted by the enzyme. This type of reactor is commonly employed for carbonic anhydrase in the laboratory to test turnover and general performance properties of the enzyme in a reactor environment. The disadvantages of this type of reactor are several-fold. Bubble chambers are inefficient in transferring gas into liquid phase. Bubbling in the system typically creates foaming of the liquid phase, which is undesirable from a process control stand point and foams are also typically deleterious to enzyme stability. The partition of the dispersed gas phase to the entrapped or immobilized enzyme is inefficient, as the reactive surface area ratio of the immobilized enzyme surface to solution phase is low. The carbonate loaded solution is recirculated, but is typically unable to achieve high loading levels without significant changes in pH, which leads to inefficient product transport. The product loads in the liquid phase combined with the other challenging issues present with bubble chambers likely preclude large scale application of these designs.

2.6. Multiphase Contactors

These reactors are in many ways equivalent to bubble chambers in the sense that the liquid and gas phases are intimately mixed in the reaction chamber in the presence of the biocatalyst (Parent et al.). The important distinction of this type of reactor is that the enzyme is bound to the solvent exposed surface of the immobilization matrix, which limits the diffusion requirements of the CO_2 and carbonate to inner portions of the immobilization support (Figure 3). These systems employ a high surface area immobilization support that is either in the form of a bead or micronized particle, which maximizes the catalyst exposure to CO_2. As with bubble chambers however, there is still a mass transfer and partition challenge of dispersing CO_2 into the liquid phase. Poorly soluble CO_2 needs to partition into an intermediary liquid phase prior to reaction with the enzyme. Because most of the CO_2 remains in a dilute gaseous environment, it along with the other gases in the stream form bubbles in the liquid solution. These bubbles not only act to prevent further CO_2 partitioning, but also create the environment for foaming within the reactor. Foams are well known to create back pressure or fouling of the plumbing of the reactor which causes spillage and process disruption. To avoid these difficulties, the reactors are often treated with antifoaming agents that add cost to the system.

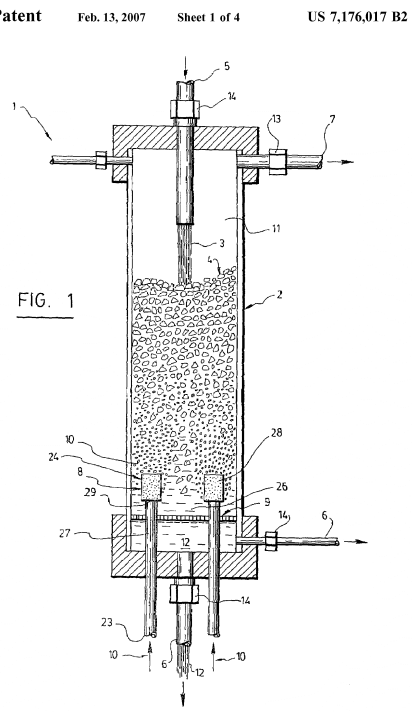

Figure 3. Multiphase contactor reactor. The diagram depicts a version of a multiphase contactor device that bubbles gas phase CO_2 into a liquid that is filled with carbonic anhydrase enzyme immobilized to a solid support. As CO_2 is converted to carbonate by the enzyme at the solid surface the solubilized ions are washed away by the liquid phase which transports the carbonate to a recovery chamber. Image obtained from US Patent 7176017.

Source: Authors

2.7. Membrane Partition Reactors

Membrane partition type reactors are a much more sophisticated and intriguing approach to reactor design (Trachtenberg 2000). In its simplest manifestation, these reactors can be viewed as a two state reaction system in which the gas phase and liquid phase are separated by a porous membrane or support (Figure 4). The enzyme is bound to the support and is exposed to the gas and liquid phases simultaneously. In this way, the partitioning of CO_2 into the liquid phase is minimized, which improves catalytic efficiency. In addition, the physical separation of the gas and liquid phases prevents foaming phenomena, as well as improves the potential loading of carbonate into the liquid phase. On the downside, these reactor membranes are often complex structures that are capital intensive to construct and replace. In addition, the surface area of the reactor is often small relative to other designs, which can limit the overall efficiency of the system.

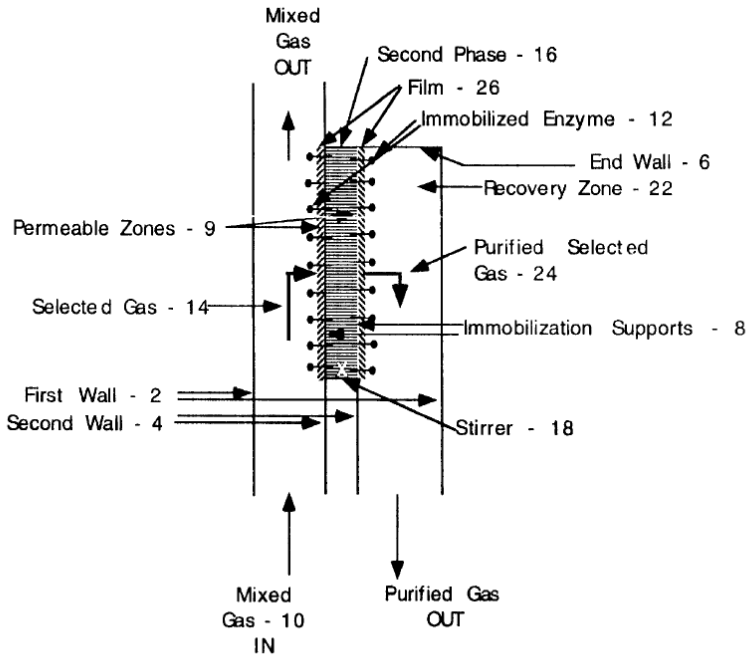

Figure 4. Membrane partition reactor. Typical description of a membrane reactor configured for trapping and concentration of CO_2. Low concentration waste stream gas enters the chamber and is reacted on the membrane surface with immobilized carbonic anhydrase. The hydrated carbonate ion is solubilized in a fluid interlayer which circulates the material to a membrane on the opposite side of the membrane chamber. Immobilized carbonic anhydrase enzyme works in reverse mode to release the CO_2 into the purified chamber for mechanical concentration and transport. Image obtained from US Patent 6143556.

Source: Authors

3.0 Cloning, Expression, and Characterization of Carbonic Anhydrase

3.1. Cloning Carbonic Anhydrase

One of the critical issues essential to demonstrating the success of this feasibility study will be to produce carbonic anhydrase in the quantities necessary for large scale application. Literature review above suggests that it is possible to produce carbonic anhydrase at the scale required to meet power plant CO_2 sequestration needs. If, as estimated above, 1 kilogram of enzyme will be sufficient to treat the CO_2 from a 300MW plant, and if the target enzyme lifetime is one working month, this would translates to only 12 kilograms of enzyme per plant per year. The cost of producing the enzyme is dependent on the scale and yield of the fermentation process. However, for the largest enzyme production processes such as applied to the cleaning industry, prices per kilogram of enzyme are well under $50. This quantity of enzyme is well within the production scope of current fermentation technology and if the price per kilogram of enzyme can approach the costs common for other commodity scale enzymes the overall cost contribution of the enzyme is well under $0.01/ton of CO_2 sequestered.

With these considerations in mind the enzyme carbonic anhydrase has been selected from a group of naturally occurring sources and will be cloned and expressed in an industrial, scale up ready system. Enzyme performance criteria and genetic engineering factors will be the driving issues that will lead to enzyme candidate selection. The successful completion of the cloning and expression portion of this feasibility project will demonstrate that a suitable carbonic anhydrase can be expressed in an appropriate industrial ready host, such as E. coli or Bacillus, and that the recombinant protein can actively ionize CO_2 in a laboratory scale reactor. These milestones will set the stage for further development to optimize the enzyme production process for larger scale demonstration processes.

3.2. Choice of Carbonic Anhydrase (CA) Enzyme

The choice of which carbonic anhydrase enzyme to work with for this project came down to several factors including:

- Preference for alpha-class enzymes
- Stability
- Resistance to inhibition
- Can be produced in large quantities
- Amenable to engineering for purification and immobilization

The preference for an alpha-class CA is derived from the desire to limit the oligomeric state of the enzyme. The greater the number of subunits an enzyme has, the more difficult it may be to stably immobilize an active enzyme. The oligomeric state of CA enzymes is homomeric, with as many as eight subunits. Alpha-class CA is usually a homodimer and typically has the least number of subunits. It has also been demonstrated that alpha-class CAs can be His_6-tagged at

their C-terminus. The desire for stability leads us to look for alpha-class enzymes from organisms whose enzymes must inherently be more stable. Enzymes from thermophilic organisms are intrinsically more stable. The enzymes from moderate thermophiles are, however, often most desirable because they strike a balance between stability and activity at the temperatures at which they are being used. Because of their exposure to a broad array of environmental conditions, extracellular enzymes are also innately more stable and resistant to inhibition.

GTI chose to work with the alpha-class carbonic anhydrase from the moderate thermophile *Streptococcus thermophilus* LMG 18311. Figure 5 shows the sequence of this enzyme. Because it is from a Gram-positive organism and carries an N-terminal signal sequence, the enzyme is predicted to be localized on the extracellular surface.

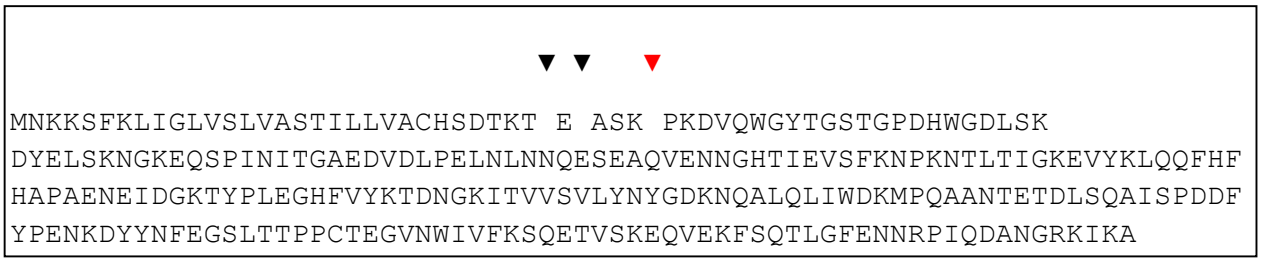

Figure 5. Sequence of the alpha-class CA from *Streptococcus thermophilus* LMG 18311, StCA-His$_6$. *Black triangles* indicate sites at which the signal peptide for export is predicted to end. The *red triangle* represents the position at which the start codon of the enzyme lacking a signal peptide was placed for expression in *Escherichia coli*.

Source: Authors

A version of the enzyme lacking its N-terminal signal peptide was cloned and placed into a stable, scalable vector for expression in *E. coli*. The signal peptide normally directs the protein to a specific location within the cell in the native organism. These signal peptides are commonly removed when cloning into *E. coli* because this organism is not capable of using the sequence. The signal peptide does not affect structure or activity as it is removed once the protein is localized in the native organism. With this vector, this enzyme could be expressed in the cytoplasm of *E. coli*, under rhamnose induction, which is a sugar molecule that acts as a signal to turn on the production of the enzyme once added to the culture. The enzyme was engineered to carry a C-terminal His6-tag which is a sequence of six histidine residues placed at the end of the enzyme. These additional residues allow for the enzyme to be purified and immobilized selectively to the bioreactor surfaces. Figure 6 is the map of the plasmid used for StCA-His6 expression.

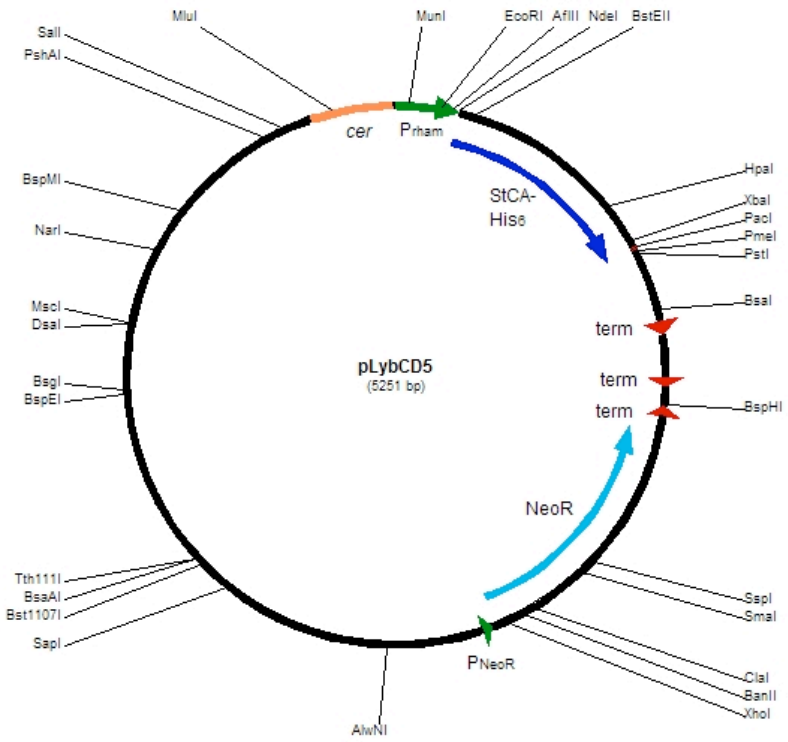

Figure 6. Plasmid pLybCD5 for the expression of StCA-His$_6$ in *E. coli*. The location of the genes for expression of *StCA-His6* and *neomycin resistance*, transcription *promoters* and *terminators*, the *cer* stabilization sequence, as well as *unique restriction sites* are labeled.

Source: Authors

3.3. Production and Isolation of Carbonic Anhydrase

Having the cloned CA genes in the appropriate plasmid vector enabled transformation of an *E. coli* K12 variant host strain that is commonly used for industrial production of proteins. The insertion of cloned plasmids converted the host *E. coli* into a system that allowed the production of carbonic anhydrase. Confirmation of successful transformation of plasmids was confirmed by growing transformed *E. coli* on petri dishes using a culture medium containing the antibiotic neomycin. Only those bacteria that host plasmids can survive in the presence of the antibiotic allowing selection of *E. coli* capable of producing carbonic anhydrase. Further confirmation of successful transformation was achieved by re-isolation of plasmid DNA from the selected candidate hosts and performing sequence analysis on the carbonic anhydrase genes housed within the isolated plasmid.

Once the expression system was validated, initial expression studies could be performed. To this end *E. coli* hosts bearing the production plasmid were inoculated into small 3 milliliter cultures and incubated overnight in a standard Luria broth culture medium at 37 °C. Cells from

these initial cultures were processed and assayed by polyacrylamide gel electrophoresis (PAGE) to determine if the CA enzyme was being produced. Analysis of these early test experiments suggested that the recombinant enzyme production system did produce reasonable quantities of CA enzyme. The fermentation was scaled up to 500 milliliters using shaking under the same conditions described for small test tube scale experiments. Several standard culture media were screened in a series of experiments and a modified culture media with the recipe shown below (Table 1) afforded reproducible yields of CA enzyme as determined from PAGE analysis.

Fermentation studies were expanded to employ 2 liter glass stirred tank fermentors using the conditions and culture media developed from shake flask studies. Cultivation in stirred tank fermentors allowed specific control of critical variables including oxygen, pH, and carbon feed. Controlling the cultivation environment allowed more efficient accumulation of biomass which enabled greater production of carbonic anhydrase. In addition to environmental control the stirred tank fermentor was configured to allow for feeding of the culture as nutrients are consumed. So called fed batch conditions are established by slowly introducing a mixture of glycerol and soy hydrolysate nutrients in a concentrated solution as these materials are used by the growing culture. In addition, the stirred tank conditions enable the actual production of enzyme to be controlled by including an inducing agent into the fermentation at a point during the culture in which the biomass has had a chance to begin growing robustly. In the case of producing carbonic anhydrase the inducer used is the sugar rhamnose. Tests were run to evaluate the cultivation time, induction time, temperature and pH of the media. The results of this initial survey revealed a set of conditions that afforded consistent high yield production of biomass and desired CA enzyme. Typical runs provided between 50 and 60 grams of wet cell mass per liter of culture over a cultivation period of 36 hours.

Once the fermentation was judged complete the biomass containing carbonic anhydrase was harvested by separating spent culture broth from biomass by centrifugation. The recovered biomass in the form of a thick paste was then subjected to primary processing during which the carbonic anhydrase was released from the cell mass by rupturing intact cells. Specifically, the biomass was dispersed in a buffer solution to stabilize pH and provide a solution to support the recovered carbonic anhydrase. The cell mass was disrupted by creating a strong shear force created by applying high pressure nitrogen gas to the cell mass. Disrupted cells and other culture debris were separated from the lysis solution by centrifugation. The remaining solution containing the CA enzyme was diluted and purified by column chromatography. In one example the enzyme was purified by passage over an affinity resin designed to selectively recognize and retain enzymes such as carbonic anhydrase that have been engineered during the cloning process to contain a defined recognition tag. The importance of the tag is not only for purification but this element is also used for initial binding during the immobilization process. The result of this selective tag based purification is shown in Figure 7. The second purification method is based upon the inherent charged nature of proteins. Each protein has a characteristic set of charges both positive and negative arising from the constituent components that make up the material. The enzyme can be separated based upon the nature of these charges from other enzymes in the mixture by passage over a resin contained in a column that has opposite charge

character from the desired protein. This so called ion exchange process enables enzymes to be isolated and purified and is a common method run on industrial scale. The PAGE image shown in Figure 8 shows the purification process of carbonic anhydrase from a crude mixture form to a stable substantially purified material that can be stored, assayed and further processed as needed.

Table 1. Culture media composition for growing recombinant *E. coli* hosts bearing the plasmid coding for carbonic anhydrase. Each of the components listed is in its respective commonly available hydrated form. Sterile culture media was prepared by combining components as indicated except for neomycin in deionized water and autoclaving for 25 minutes at 121°C and 15psi. Once the solution had cooled the neomycin solution was added aseptically. pH of the culture broth is adjusted to 6.8 prior to inoculation with sterile 1N hydrochloric acid or concentrated ammonium hydroxide.

Component	Concentration per liter of culture broth
Ammonium phosphate	2 grams
Potassium phosphate	6.75 grams
Citric acid	0.85 grams
Glycerol	20 ml (50% wt)
Yeast extract	2 grams
Pea hydrolysate	2 grams
Magnesium sulfate	2 ml (1.4 molar solution)
Trace elements	1.25 ml
Neomycin	1ml

Trace metal component	Concentration per liter of water
Sulfuric acid	0.5 ml
Manganese sulfate	2.28 grams
Zinc sulfate	0.5 grams
Boric acid	0.5 grams
Copper sulfate	25 mg
Sodium molybdate	25 mg
Cobalt chloride	45 mg

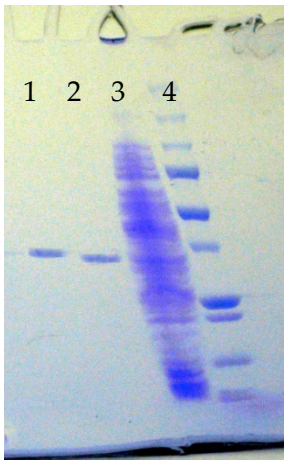

Figure 7. Expression and purification of carbonic anhydrase. Lanes are described left to right. Lane 1. Standard protein sample containing 6xHis Tag (36Kd). Lane 2. Purified carbonic anhydrase 6xHis Tag enzyme using affinity chromatography. Lane 3. Crude lysate from carbonic anhydrase expression trial. Lane 4. Standard protein samples of known relative size.

Source: Authors

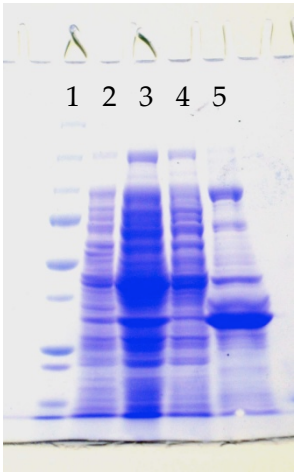

Figure 8. Purification results for CA isolation. Lanes are described left to right. Lane 1. Molecular weight standard protein samples. Lane 2. Crude lysate sample. Lane 3. Eluted samples from DEAE column. Lane 4. Wash material from affinity column. Lane 5. Initial purification sample non optimized affinity column.

Source: Authors

Once the enzyme was processed, purified, and stored, the question of enzyme activity was addressed. There are two methods commonly used to examine the functional activity of carbonic anhydrases. The first involves a surrogate substrate that changes color when reacted with the enzyme (Verpoorte et al. 1967). This substrate known as p-nitrophenyl acetate is degraded in the presence of the enzyme and produces a yellow color in solution. The color can be monitored over time in a spectrophotometer to afford quantitative assessment of the enzyme's activity. This method while simple is limited by the fact that the substrate is not similar to carbon dioxide and direct comparisons to predict activity behavior between the two materials in cannot be made. The more complex but directly applicable assay method involves measuring the change in solution pH as carbonic anhydrase reacts with carbon dioxide (Wilbur and Anderson 1948; Silverman and Lindskog 1988). The catalytic reaction hydrates the carbon dioxide molecule creating bicarbonate ions. The bicarbonate in solution causes the pH of the overall solution to become acidic which is manifest as a reduction in pH as indicated by a pH monitor. This method while more technically involved is a direct measure of carbonic anhydrase activity and is also the primary method used for evaluating the function of the biosequestration reactor described in Chapter 4.

Assays involving the colored substrate surrogate and carbonic anhydrase were set up by dissolving a small amount of enzyme into a buffered solution and then adding the substrate. The reactions were set up in cuvette that was then placed in a spectrophotometer and the change in color monitored over time. The assay experiment indicated that unlike other carbonic anhydrase enzymes, the cloned carbonic anhydrase from *Streptococcus thermophilus* LMG 18311 did not readily accept the surrogate substrate for reaction. The reaction proceeded very slowly and overall was considered to be of limited use for determining enzyme action. The carbon dioxide hydration assay was set up by preparing a saturated solution of carbon dioxide in water. This substrate solution was then added to a stirred, weakly buffered solution containing carbonic anhydrase and the pH of the solution was monitored over a defined time period. The data obtained were corrected for the spontaneous uncatalyzed hydration of carbon dioxide which was measured in a similar manner except that the enzyme was left out of the assay solution. Figure 9 shows the respective data for the catalyzed versus uncatalyzed reactions. Calculations from the collected data indicate that the cloned carbonic anhydrase in this study hydrates carbon dioxide at a rate of $2x10^9$ per sec which is consistent with literature values for other carbonic anhydrase enzymes.

Demonstration that a cloned carbonic anhydrase could be produced, purified and shown to have high activity allowed for further work to progress whereby the enzyme would be placed into a bioreactor system to study the feasibility of using the enzyme as a carbon dioxide sequestration catalyst.

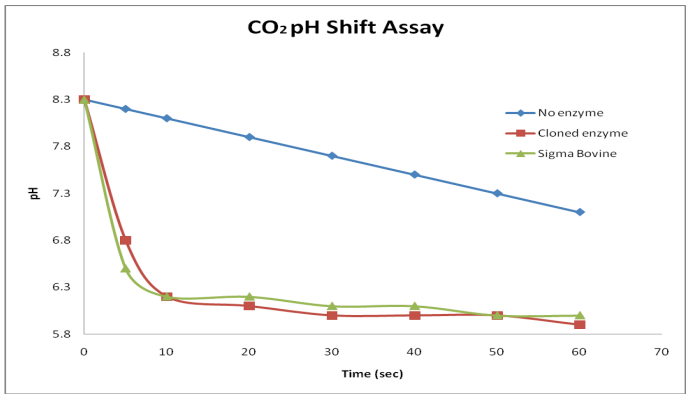

Figure 9. The assay of purified cloned carbonic anhydrase. The change in pH of a 10 mM CO_2 solution in the presence of 0.1 micrograms per milliliter solution was monitored by pH meter. Data is an average of 3 independent experiments. Enzyme catalyzed reactions are compared to the spontaneous rate of hydrolysis recorded for solutions without added enzyme. Positive control experiments substituted commercial carbonic anhydrase from bovine erythrocytes supplied by Sigma-Adrich Chemical Company.

Source: Authors

3.4. Immobilization of Carbonic Anhydrase

The approach to CO_2 sequestration using a biomimetic process aims to remove CO_2 from the gaseous state and hydrate the molecule as bicarbonate ion that is soluble in water. Once in an ionic form the bicarbonate can be removed by precipitation as a solid that is recovered and deposited as a stable material. Water solvent is the principal vehicle for transporting bicarbonate through the bioreactor system and as such this poses a challenge of retaining the enzyme catalyst in the reaction zone. Carbonic anhydrase like many enzymes is a water soluble material that that will exist in the water phase of the reactor and be washed out of the system if not otherwise confined within the reactor. The concept of enzyme immobilization has been extensively utilized in enzyme catalyzed systems in order to prevent enzymes from being lost in reaction vessels.

In the case of this study the objective is not only to prevent loss of the enzyme by washout but also to position the catalyst in a specific position to allow for maximal interaction with both the gaseous carbon dioxide and the water solvent needed to remove bicarbonate products. Since the ultimate application is industrial scale cost of processing and immobilizing the carbonic anhydrase is a critical concern. The preferred approach would be to be able to purify the enzyme from a crude culture preparation which enables a high level of active enzyme on a surface and bind to the bioreactor ready support in a single step. This single step process is possible with the cloned carbonic anhydrase in this study since the enzyme has been engineered to include a binding tag. This tag was used earlier in the study to purify the enzyme during initial characterization experiments. The same binding tag can be used to stick the enzyme to a reactor surface and prevent washout during the sequestration process. Another consideration in

preparing the binding surface is to utilize a low cost robust material that can be readily inserted into a reactor that not only holds the enzyme in place but also can serve as an interface between the gas phase containing carbon dioxide and the water solvent that will remove the bicarbonate product.

A woven silica fabric is an ideal material that meets the necessary criteria needed as an immobilization support for this study. The fabric material can be treated with a chemical coating that specifically binds the engineered tag of the cloned carbonic anhydrase enzyme. To this end small coupons of model silica woven fabrics were treated with a proprietary coating reagent developed previously in the laboratory. To the treated surfaces was added a solution containing the cloned carbonic anhydrase which was given the opportunity to bind to the coated surface. The enzyme treated surface was then washed with a weak buffer solution to remove excess and unbound enzyme from the surface. The activity of the enzyme was then tested with both assays described above. For the surrogate substrate, small samples of the treated woven fabric were submerged in a buffered solution that contained the substrate. Experiments that contained the carbonic anhydrase bound to the coated surface showed a slow evolution of color indicating the presence of active enzyme bound to the surface. Control experiments that included coated fabric without enzyme bound to the surface showed no color development. The experiment was repeated once per day on the same fabric samples over the course of 4 days to demonstrate that the enzyme remained bound to the surface and that the carbonic anhydrase was stable when bound (Figure 10). A second set of experiments was performed that utilized the pH shift assay and carbon dioxide as the substrate. In these experiments rapid shifts in pH were observed compared to separate experiments that measured spontaneous carbon dioxide hydration using fabric samples without enzyme.

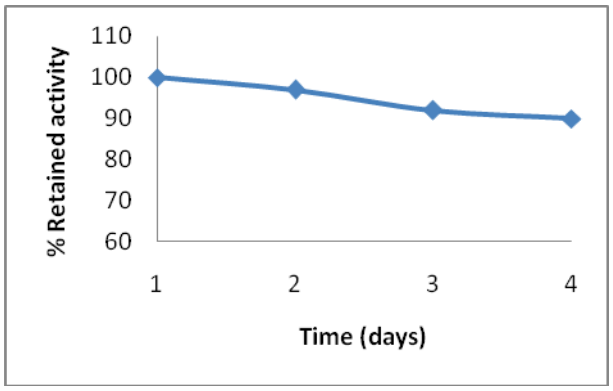

Figure 10. Activity retention of immobilized carbonic anhydrase. The enzyme bound to the coated woven silica fabric was tested in a pH shift assay over the period of 4 days. Between tests the test fabric was washed with buffer and stored as a wetted material in buffer at room temperature. The retained activity is defined as the rate of pH shift observed relative to the activity recorded on Day 1.

Source: Authors

While the data indicated that the enzyme catalyzed the hydration of carbon dioxide it became apparent that the rate of pH shift was slower than expected for the amount of enzyme believed bound to the fabric surface. The wash solution following the initial binding process was tested for residual carbonic anhydrase activity. The amount of enzyme recovered in the wash solution indicated that the original binding of the enzyme to the surface was indeed 40% lower than expected. There are a number of factors that can influence binding to the surface including the composition of the aqueous buffer solution used for binding, the composition of the wash solution, and the level of binding capacity on the coated surface. Enhancing the yield of coated surface binding will be one of the subjects during the future optimization phase of the program. In spite of the lower than expected surface binding of carbonic anhydrase to the coated woven fabric, the overall activity of the surface was sufficient to proceed to bioreactor construction and feasibility testing.

4.0 Bioreactor Design and Testing

Carbon dioxide sequestration using a bio-mimetic process poses a number of significant challenges unique to working with biological components. Unlike subterranean or deep water storage or conventional chemical conversion technologies, incorporating a bioreactor operates at ambient temperatures, pressures with no harsh or toxic chemicals. The limitations placed on operating conditions require a strong emphasis on the reactor design. Other attempts to create scalable bioreactors have faced challenges in managing access of carbon dioxide to the enzyme, stabilization of enzyme in the system and managing fluid/gas interfaces. In this study, the approach to reactor design began with the realization that the enzyme can function efficiently at a gas/water interface. This realization is the centerpiece for the reactor design and clearly differentiates this system from predecessors found in the literature.

4.1. Bioreactor Construction

The bioreactor was designed as a laboratory scale prototype device that could effectively incorporate the anticipated functional elements of a larger working device envisioned for future development. As shown in Figure 11, the key components of the reactor are the fluid and gas handling system and the solid support interface material that acts to hold the carbonic anhydrase enzyme in place for reaction with gaseous CO_2 while allowing moisture to exchange with the surface. Studies described in the previous chapter established that a woven fiberglass material coated with a selective binding material could recognize, immobilize, and provide a stable support for the cloned carbonic anhydrase enzyme. Furthermore the woven fiberglass fabric material has had a long history in liquid phase filtration and high air flow bag house applications. These latter filtration based uses of this type of fabric establish that the material can function in a wide variety of environments and serve to separate different environments. In addition, woven fiberglass fabrics are used on very large scale and are among the most robust cost effective substrates used in industrial applications.

The bioreactor is designed as a modular device that is composed essentially of two chambers separated by a woven silica fabric "membrane." Each frame chamber is built from polyethylene stock and covered by a piece of acrylic sheet. The interface between the chambers is lined with a watertight soft rubber gasket that holds the membrane in place. The chambers are held together by regularly spaced bolts. The reactor is plumbed by drilling holes into the frame at the appropriate locations to allow for fluids or gases to pass into or out of the device. The falling film of liquid is created by a silicone dispersion tube at the top of the liquid chamber. The dispersion tube allows a film of liquid to spread across and down the surface of the membrane on the liquid chamber side. The liquid falling off the membrane is collected at the bottom of the liquid chamber and pumped to the recycling reservoir. In the gas phase chamber CO_2 rich gas enters from the bottom port through silicone tubing. The gas interacts with the carbonic anhydrase loaded membrane and the CO_2 is converted to bicarbonate which is carried away by the liquid saturating the membrane. CO_2 free gas is then allowed to escape at the top of the reactor.

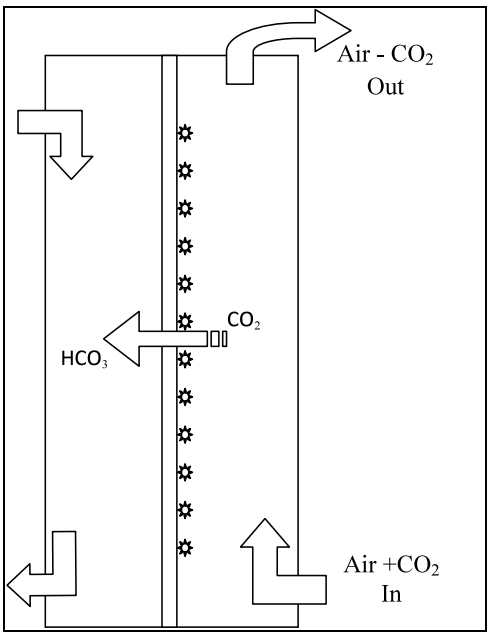

Figure 11. Conceptual bioreactor design. The critical elements of the bioreactor include a two chamber system. The chambers are separated by a membrane that is loaded with carbonic anhydrase. The right chamber allows a CO_2 rich gas to flow and react with the bound enzyme at the membrane surface. The left chamber contains a falling film of aqueous buffer solution to wet the membrane and to carry the bicarbonate product away from the reaction chamber.

Source: Authors

The bioreactor was assembled as shown in Figure 12 and was plumbed with a peristaltic type pump to provide fluid flow to create and maintain the falling film in the liquid chamber. Start up testing of the reactor was performed by turning on the pump using water for the liquid chamber. The pump was tested over a range of flow rates from 1 to 100 milliliters per minute, which wetted the reactor surface material without flooding the liquid side of the chamber. While the fabric surface was wetted efficiently, there was no observed liquid breakthrough to the gaseous chamber indicating the fabric was able to separate gas from liquid phase effectively. A nitrogen stream from a compressed gas cylinder was used as the model gas flowing through the gas chamber of the reactor. As with the liquid phase testing, there was no observed gas breakthrough across the reactive surface between 0.1 and 2 liters per minute gas flow. With stress testing complete the reactor was ready commencement of CO_2 reactions.

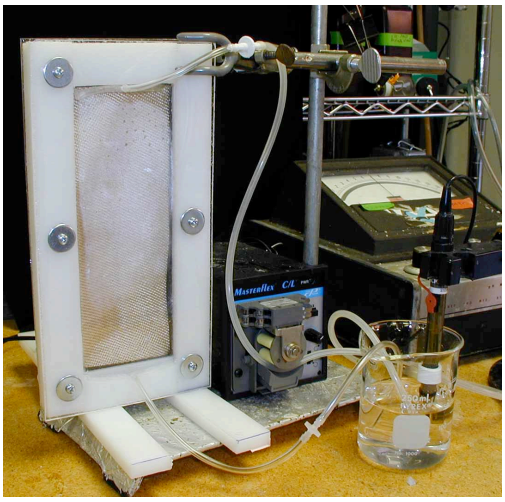

Figure 12. Photograph of prototype laboratory sequestration bioreactor. Note the plumbing that allows for recycling from the liquid storage to the top of the reactor, establishing the falling liquid film across the reactive material surface. Included in the figure is a pH probe positioned in the liquid storage for analysis of the liquid pH as CO_2 is hydrated from the gaseous phase into the liquid phase by the carbonic anhydrase bound to the reactive surface.

Source: Authors

4.2. Bioreactor Testing

The key aspect of the bioreactor is to determine the effectiveness of the woven silica fabric surface. The fabric has two principle functions: to act as a physical barrier between the liquid phase and gas phase containing the CO_2, and to immobilize the carbonic anhydrase enzyme to the surface. The immobilization function is critical for holding the enzyme in position so that it can catalyze the chemical reaction responsible for converting the CO_2 to carbonate. The bioreactor was designed as a laboratory scale prototype device that could effectively incorporate the anticipated functional elements of a larger working device envisioned for future development. From previous studies it was demonstrated that a woven fiberglass material could be engineered with a coating that could then be loaded with carbonic anhydrase. The consequent material demonstrated reasonable enzyme activity that was stable over an extended period of time. Initial testing of the surface in place within the bioreactor established that the surface could be wetted without liquid or gas breakthrough over the range of flow rates expected during operation.

The functional testing of the bioreactor was performed initially with the woven fabric set in the reactor without enzyme loaded on the surface. This set of experiments was designed to establish the control operating environment and set the baseline rate for spontaneous hydration

of carbon dioxide in the system. To measure the hydration of CO_2 in the reactor the falling film solution, composed of a weakly buffered water solution, was collected in a reservoir fitted with a pH monitor. The reservoir was stirred to make sure that the solution was mixed and the container was kept covered to prevent atmospheric CO_2 from introducing pH shifts resulting in non-bioreactor related phenomena. The pH monitoring process is similar to the pH shift assay described earlier and uses the same water buffering system, although the configuration is altered to account for the larger volumes of fluid needed to create the falling film in the bioreactor. The gas phase chamber of the bioreactor was purged with nitrogen gas from a cylinder and then the liquid pump on the bioreactor was started. The pH was monitored in the reservoir while the pump and gas were flowing over a period of 10 minutes to determine if the system could maintain a constant pH with essentially no carbon dioxide introduced. No pH changes were observed during the operation under a nitrogen gas purge in the gas chamber of the reactor.

The satisfactory results using nitrogen in the gas chamber led to a further set of control experiments in which the gas introduced and purged through the device was composed of carbon dioxide supplied by a cylinder. As with the nitrogen experiments, the woven fabric surface in the reactor was not loaded with carbonic anhydrase. When the reactor was started the pH of the reservoir solution showed a slow decrease over the 10 minute run time going from a starting pH of 8.3 to 8.0. The rate of decrease was much slower than previous liquid phase pH shift assays and can be potentially attributed to a number of factors. One explanation for the slow change in pH could be that the greater overall liquid volume of the system requires a reasonable quantity of CO_2 hydration to cause a recordable change in the reservoir solution. Additionally, the woven fabric creates a barrier that may limit the amount of carbon dioxide that physically contacts the free liquid phase, thereby slowing down the observed spontaneous hydration of CO_2 in the system. Regardless of the cause, the rate of background carbon dioxide hydration is relatively slow and provides an opportunity to be able to record any acceleration in carbon dioxide hydration.

In the final set of experiments the bioreactor was set up with the woven fabric loaded with carbonic anhydrase. The gas phase chamber was purged with carbon dioxide and the liquid flow was started. The pH measured in the reservoir dropped at a faster rate than the control experiments and continued to decrease during the operation time course of the experiment as shown in Figure 13. As with the control experiment using carbon dioxide in the gas phase without enzyme loaded on the fabric surface the rate of pH change was considerably slower then observed in pH shift assays with either free enzyme or enzyme bound to small sample fabrics. Accounting for the difference in surface area between the small sample tests and the bioreactor as well as the loading levels of the enzyme on the surface leads to an estimation of the rate effect of the permeation of gas to the surface and the resulting rate of bicarbonate product to pass through the woven fabric. In agreement with literature precedent (Cowan et al. 2003), the nature of the interface plays a significant role in the overall rate of product recovery in the liquid phase. It is likely that the movement of product bicarbonate through the fabric layer is the overall rate limiting process operating within the bioreactor.

The results from the bioreactor trials establish that it is feasible for a multiphase bioreactor to successfully sequester carbon dioxide from the gas phase. The use of the enzyme carbonic anhydrase significantly increases the rate at which gas phase carbon dioxide is hydrated to bicarbonate ion. The configuration of the bioreactor in principle is able to enable the capture, conversion and recovery of carbon dioxide into a buffered water solution. The trial experiments also demonstrate several opportunities for enhancing the bioreactor that would be expected to allow for further scale up. These changes include improving the binding yield of the carbonic anhydrase to the surface coating and altering the fabric material to allow more efficient transfer of product bicarbonate ion into the buffered water solution. In addition, a larger scale device would enable a better understanding of the operational properties of the bioreactor and provide more precise and quantitative data that would be required for economic modeling of the system. The economic modeling would provide insight into the prospects of the overall costs associated with set up and operation of this type of bioreactor on a pilot scale and beyond.

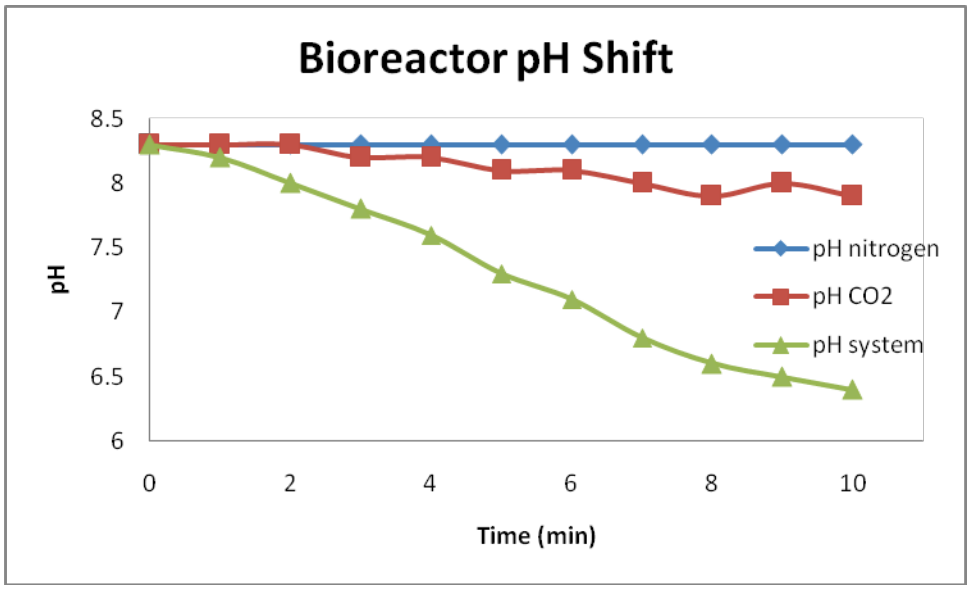

Figure 13. Plot of bioreactor pH shift. The bioreactor was run under different conditions and the change in the pH of the liquid resevoir measured over time. Blue line is data collected from the reactor with no enzyme and gas chamber purged with nitrogen. Red line depicts data collected for the bioreactor gas chamber purged with CO_2 and a surface containing no enzyme. Green line represents data collected from the bioreactor running with CO_2 purged in the gas chamber and the fabric surface loaded with carbonic anhydrase.

Source: Authors

5.0 Conclusions and Recommendations

5.1. Conclusions

The state of art review demonstrates that the principle of enzyme-based biosequestration is a viable green alternative for removing CO_2 from the environment. The results from the preliminary bioreactor trials also established that it is feasible for a multiphase bioreactor to successfully sequester CO_2 to bicarbonate ion using enzyme carbonic anhydrase. It is expected that as these processes mature from the laboratory and pilot plant stage and into demonstration scales, the practical implementation of these technologies will become a reality. An economic analysis of biocatalytic sequestration has yet to be developed and will likely depend on the collection of data from larger scale demonstration of various reactors installed in real applications. In spite of limited application knowledge, current understanding of biocatalytic sequestration suggests that this approach to managing CO_2 waste streams warrants further evaluation and development.

5.2. Recommendations

This project has focused on two of the three aspects that are critical to CO_2 sequestration using biocatalytic methods, the enzyme and the bioreactor. However, a third component to sequestration which involves the removal and processing of the sequestered CO_2 has to be addressed in the future. There are essentially two current options to the question of what to do with the carbon once it is removed from the flue gas stream. Since many of the bioreactors that have been developed concentrate on generating carbonate ions from CO_2 using carbonic anhydrase, it stands to reason that the logical step would be to remove the carbonate from the liquid phase. Many carbonate salts, particularly magnesium and calcium carbonates, are materials that are poorly soluble in water and constitute arguably the largest repository form of CO_2 on the planet. Estimates for this carbon pool are approximately 1.5×10^{17} metric tons of CO_2 that is held as insoluble matter on Earth (Dunsmore 1992). Carbonate removal from aqueous solutions can be thought of in terms of a relatively straightforward precipitation and filtering process of the liquid stream. Mixing calcium or magnesium ions with the aqueous carbonate creates an insoluble salt that can be harvested and then transported as a stable non-hazardous material. The real challenge is actually two-fold: introduction of the counter ions and then harvesting and disposing of the solids that are formed.

On laboratory scale, these issues are very straightforward and model systems have demonstrated that carbonate in liquid streams can efficiently be precipitated and removed from the solution. More impressively a number of bioreactor systems have demonstrated that the process can be run continuously with the carbonate free liquid re-circulating back to the bioreactor to collect more carbonate (Blais et al 2003). The challenge becomes more apparent when laboratory scale systems are modeled for large scale applications. In this case, it is important to consider the volumes and amounts of materials needed for carbonate removal and the consequent amounts of final solids that need to be transported. Consider that the mass of carbonate salt solids is typically between 7 and 8 times the mass of the original mass of the coal burned. If a nominal 300MW coal fired power plant consumes 100 tons of coal per hour, it will

generate between 700 and 800 tons of solid carbonate salts per hour. The question involves identifying the source of the counter ions that would meet these quantities of supply. Suggestions of using brines from seawater have been proposed and while the quantity of water needed is large, it is not so onerous as to be completely unrealistic (Bond et al 2001). Perhaps more reasonable is the notion of using an ion exchange strategy in which magnesium or calcium laden minerals are used as an exchange bed and filter system that would strip the carbonate from solution as it is passed though the bed. While this approach has not been demonstrated, it would be equivalent to commercial and municipal percolation beds used in waste water treatment.

Another alternative would be a derivative of technology under development and sponsored by the United States Department of Energy (Huijgen and Comans 2004). Current thinking is that CO_2 can be concentrated and transported by pipeline for long term storage in subterranean facilities. The challenge with this approach, beyond the choice and availability of appropriate and safe locations, is the cost associated with concentrating and purifying CO_2 from mixed dilute flue gas streams. The requirement for concentration and storage of CO_2 is greater than 95% purity, which poses significant problems for process engineering. The dual function membrane bioreactor has been developed that employs carbonic anhydrase as selective catalyst that is capable of removing CO_2 from dilute gas streams. Process modeling based on data from laboratory scale trials suggests that the membrane bioreactor system can significantly reduce the cost of CO_2 concentration compared to currently available conventional technologies. What remains to be determined is whether the enzyme production costs can be improved to economically viable levels.

In short, the major unmet challenges in biocatalytic sequestration of CO_2 are the type of enzyme used and the secondary processing of carbonate products. The former issue will require continued search for enzymes that have desirable characteristics such as thermotolerance, osmotolerance and low production costs. The concern over secondary processing involves identification of methods and materials that will enable efficient capture and recovery of the carbonate product from bioreactor solutions. The expansion of mass created by formation of carbonate and its salts and the nature of the likely sources of counter ions for precipitation of carbonates is likely to be an ongoing challenge for the foreseeable future. In spite of the remaining hurdles associated with biocatalytic CO_2 sequestration, there is enough evidence to suggest that these processes be considered seriously as complementary approaches to CO_2 removal from the environment.

5.3. Benefits to California

California has emerged as a leader in developing environmental policy in the United States. Its large population, unique geographic features and diversity of natural wonders create challenges for the state to balance utilization of resources, commerce, recreation and responsible stewardship of lands. Pollution of air, land and water is a persistent consequence of large population densities and robust economic activity that needs ambitious intervention to avoid profound damage to California's environment. No pollutant is quite as insidious as carbon

dioxide. The gas is odorless, colorless and historically viewed as a relatively benign material that is pumped into the air and taken away. Recent evidence suggests that carbon dioxide may actually play a larger active role in the environment. Not only is the gas needed by photosynthetic organisms for growth but at high levels it may contribute to significant changes in weather that can adversely affect the normal functioning of the environment.

Human activity in particular has been cited as a primary mechanism for the dramatic increase in atmospheric concentrations of CO_2. The delicate balance and interplay between human lifestyle, economic activity and environment is perhaps nowhere in the world better exemplified than in the state of California. The state has recognized the role of responsible environmental stewardship and is cognizant of the need for introducing innovative means to combat pollution and specifically CO_2 emissions. The use of biosequestration technologies to combat progressive increases in CO_2 emissions represents a green alternative that can serve as a permanent, safe, environmentally friendly means to sequester CO_2 from point sources of emission. As with other pollution remediation issues, CO_2 minimization offers only intangible benefits to commerce in terms of bottom line value proposition. Government intervention is needed as a change agent to compel both commercial interests as well as the residents to recognize the overall benefits of environmental responsibility. Actions already underway within California provide means that will allow sequestration and CO_2 emissions management to become reality. The efforts of this project provide the opportunity to add a practical tool to enable point source CO_2 emissions sites to economically implement CO_2 sequestration programs.

6.0 References

Alber, B.E. and J.G. Ferry, "A Carbonic Anhydrase From the Archeon *Methanosarcina thermophila*." *PNAS*, 1994, 91, 6909-6913.

Al-Rawajfeh, A.E., *Dissertation: Modeling and Simulation of CO_2 Release in Multiple Effect Distillers for Seawater Desalination*. Department of Engineering Sciences, University of Halle-Wittenberg, Germany, 2004.

Arnold, F.H. and A.A. Volkov, *Curr. Opin. Chem. Biol.* 1999, 3, 54-59.

Barth et al., "Kinetics and Mechanisms of the Reactions of Carbon Dioxide with Alkanolamines; A Discussion Concerning the Cases of MDEA and DEA," *Chemical Engineering Science*, 1984, 39, 1753-1757.

Benevides, B.C.C., C. Mateo, A.V. Carrascosa, A.Vian, J.L. Garcia, G. Rivas, C. Alfonso, J.M. Guisan, and R. Fernandez-Lafuente, "One Step Purification, Covalent Immobilization and Additional Stabilization of a Thermophilic Polyhistagged B-galactosidase From *Thermus* sp. Strain T2 by Using Novel Heterofunctional Chelate Epoxy Sepabeads," *Biomacromolecules*, 2003, 4, 107-113.

Blais, R. et al. U.S. Patent no. 6524843, 2003.

Boller, T., C. Meier, and S. Menzler, "Eupergit Oxirane Acrylic Beads: How to Make Enzymes Fit for Biocatalysis," *Org. Proc. Res. Dev.* 2002, 6, 509-519

Bond, G.M., J. Stringer, D.K. Brandvold, F.A. Simsek, M.G. Medina, and G. Egeland, "Development of Integrated System for Biomimetic CO_2 Sequestration Using the Enzyme Carbonic Anhydrase," *Energy & Fuels* 2001, 15, 309-316.

Cowan, R.M., J.J. Ge, Y.J. Qin, M.L. McGregor, and M.C. Trachtenberg, "CO_2 Capture by Means of an Enzyme-Based Reactor," *Ann. NY Acad. Sci*. 984:453-470, 2003.

Daigle, R. et al., U.S. Patent appl. 20060257990.

Department of Energy, *Carbon Sequestration: State of the Science*, DOE, Washington, D.C. 1991.

Dunsmore, H.E. "A Geological Perspective on Global Warming and the Possibility of Carbon Dioxide Removal as Calcium Carbonate Mineral," *Energy Conversion and Management*, 1992, 33, 565-572.

Electric Power Research Institute. Electricity Technology RoadMap, Powering Progress, 1999 Summary and Synthesis. EPRI, Palo Alto, CA.

Energy Information Administration. (n.d.). Retrieved from http://www.eia.doe.gov/environment.html

Faber, K., *Biotransformations in Organic Chemistry*. Springer-Verlag, New York, 1992 pp.1-20.

Forsman C., "Plant Carbonic Anhydrases: Structure and Mechanism." *EXS.* 2000, 90, 519-33.

Forsman, C., G. Behravan, A. Osterman, B.H. Jonsson, "Production of Active Human Carbonic Anhydrase II in *E. coli*.," *Acta Chem Scand B.* 1988, 42(5):314-8.

Fujii et al. *Patent No. 6,274,108*. U.S.

Gerrard, W., *Solubility of Gases and Liquids*, Plenum Press, New York, 1976.

Huijgen, W.J.J. and R.N.J. Comans. *Carbon Dioxide Sequestration by Mineral Carbonation*. Energy Research Center of the Netherlands, 2004, publication ECN-C-5-022

Khalifah, R. and Silverman D.N., *Carbonic Anhydrase Kinetics and Molecular Function, The Carbonic Anhydrase*, Plenum Press, New York, 1991, pp. 49-64.

Kroschwitz, J.I., and M. Howe-Grant, *Reactor Technology in Encyclopedia of Chemical Technology*, fourth edition, vol. 20, John Wiley & Sons, New York, (1996), pp. 1010-1011.

Lal R., "Sequestration of Atmospheric CO_2 in Global Carbon Pools." *Energy Environ. Sci*., 2008, 1, 86-100.

Letisse, F., S. Lamare, M. Legoy, and M. Graber, "Solid/Gas Biocatalysis: An Appropriate Tool to Study the Influence of Organic Components on Kinetics of Lipase Catalyzed Alcoholysis." *Biochem. Biophys. Acta*, 2003,1652, 27-34.

Lindskog, S., "Carbonic Anhydrase," In: *Zinc Enzyme*, T.G. Spiro (ed), Wiley-Interscience Publications, John Wiley & Sons, (1983), pp. 77-210 .

Maren, T.H., C.S. Rayburn, and N.E. Liddell, "Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Implications," *Science*. 1976, 191, 469-472.

Martinek, K., and V.V. Mozhaev, "Immobilization of Enzymes: An Approach to Fundamental Studies in Biochemistry." *Adv Enzymol Relat Areas Mol Biol*. 1985, 57, 179-249.

Meldrum, N.U., and F.J Roughton, "Carbonic Anhydrase. Its Isolation and Properties." *J. Physiol*. 1933, 80, 113-142.

Montero, S., A. Blanco, M.D. Virto, L.C. Landeta, I. Agud, R. Solozabal, J.M. Lascaray, M. de Renobales, M.J. Llama, and J.L. Serra. **"**Immobilization of *Candida rugosa* Lipase and Some Properties of the Immobilized Enzyme," *Enzyme Microb. Technol*., 1993, 15, 239-247.

Parent, C. et al., *Patent No. 7,176,017*. U.S.

Pastorekova S., S. Parkkila, J. Pastorek, and C.T. Supuran, "Carbonic Anhydrases: Current State of the Art, Therapeutic Applications and Future Prospects." *J Enzyme Inhib Med Chem*. 2004 ,19,199-229.

Pinola et al. "Simulation of Pilot Plant and Industrial CO_2 — MEA Absorbers," *Gas Separation & Purification*, 1993, 7.

Porath, J., J. Carlsson, Y. Olsson, and G. Belfrage, "Metal Chelate Affinity Chromatography, a New Approach to Protein Fractionation." *Nature*, 1975, 258, 598-599.

Premkumar, L., H.M. Greenblatt, U.K. Bageshwar, T. Savchenko, I. Gokhman, J.L. Sussman, and A. Zamir, "Three-Dimensional Structure of a Halotolerant Algal Carbonic Anhydrase Predicts Halotolerance of a Mammalian Homolog." *Proc.Natl.Acad.Sci.Usa* 2005*102, 7493-7498*

Ramachandran, P.A. and R.V. Chaudhari, *Three-Phase Catalytic Reactors*, Gordon and Breach Science Publishers, Philadelphia, Introduction, 1983, pp. 1-14.

Sartori, G. et al., *Patent No. 4,112,052*. U.S.

Schmalensee R., T.M. Stoker, and R.A. Judson, "World Carbon Dioxide Emissions: 1950–2050," *The Rev. Econ. Stat*., 1998, 80, 15-2.

Silverman, D. and S. Lindskog, "The Catalytic Mechanism of Carbonic Anhydrase: implications of a Rate Limiting Protolysis of Water," *Accts Chem. Res*. 21, 2588, 1988.

Silverman D.N. and S.H. Vincent, "Proton Transfer in the Catalytic Mechanism of Carbonic Anhydrase." *CRC Crit Rev Biochem*. 1983, 14, 207-55.

Simsek, F.A., G.M. Bond, J. Stringer, "Immobilization of Carbonic Anhydrase for Biomimetic CO_2 Sequestration." *J. World Res. Rev*., 1999, 11, 603.

Smith K.S. and J.G. Ferry, "Prokaryotic Carbonic Anhydrases." *FEMS Microbiol Rev*. 2000, 24,335-66.

Spelman, J., S. Skrien, and T.E. Parker. "Power Plant Exhaust Gas Simulator for the Development of Optical Diagnostics," *Rev. Sci. Instrum.* 2001, 72, 3699.

Suchdeo S.R. and J.S. Schultz, "Mass Transfer of CO_2 Across Membranes: Facilitation in the Presence of Bicarbonate Ion and the Enzyme Carbonic Anhydrase," *Biochim. Biophys. Acta*, 352 1974, 412-440.

Suzuki et al. *Patent No. 6,051,161.* U.S.

Trachtenberg, M., U.S. appl. 20080003662.

Trachtenberg, M., U.S. *Patent No. 6,143,556*, 2000.

Treybal, Robert E., *Mass Transfer Operations*, Third Edition, McGraw-Hill, New York. Part-2: Gas-Liquid operations Chap. 6: Equipment for Gas-Liquid Operations pp. 197-217 and Chap. 8 Gas Absorption, 1980, pp.275-341.

Tripp, B.C., K. Smith, and J.G. Ferry, "Carbonic Anhydrase: New Insights for an Ancient Enzyme." *J Biol Chem*. 2001, 276, 48615-8.

Verpoorte J.A., S. Mehta, and J.T. Edsall, "Esterase Activities of Human Carbonic Anhydrases B and C," *J. Biol. Chem*. 1967, 242, 4221-4229.

Wilbur, K. and N. Anderson, *J. Biol. Chem*. 176, 147, 1948.

Yegulalp, T.M., K.S. Lackner, and H.J. Ziock, "A Review of Emerging Technologies for Sustainable Use of Coal for Power Generation," *International Journal of Surface Mining Reclamation and Environment* 2001, 15, 52-68.

Yoshida et al. *Patent No. 5,603,908*. U.S.

7.0 Glossary

Biocatalysis	A method of running chemical reactions with the assistance of a biological molecule such as an enzyme.
Carbon Fixation	A process by which carbon is transformed from one state to another. Typically, carbon (as carbon dioxide) is reduced to a solid form of material such as performed by plants.
CO_2	A fully oxidized form of carbon that exists as a gas at normal temperatures and pressures. It is odorless and colorless and is believed to be a contributor to global warming as a greenhouse gas.
DNA	An acronym standing for deoxyribonucleic acid, commonly viewed as the molecule of life. The material is a biopolymer of 4 key nucleic acids: adenine, guanine, thymine, cytosine that represent the key building blocks of living organisms.
Electrostatic	An interaction in which charged or partially charged molecules are able to be attracted to or repelled from one another.
Enzyme	A biopolymer composed of amino acids linked together in a defined sequence as defined by corresponding DNA template. The biopolymer is folded into discreet structures that accelerate specific chemical reactions to occur.
Genetic Engineering	Methods, procedures, and technologies that permit direct manipulation of genetic material in order to alter the hereditary traits of a cell, organism, or population.
Halophile	An organism that requires a salty environment.
Henry's Law	The principle that at equilibrium the amount of gas dissolved in a given volume of liquid is directly proportional to the partial pressure of that gas in the gas phase.
Hydroxyl	A functional group in a molecule that contains an oxygen atom and a hydrogen atom bonded together as in alcohols.
Kilodalton	The Dalton is a measurement of the approximate mass of one hydrogen atom. The measurement is not as precise as molecular mass but is often used interchangeably particularly in biology. Kilodalton is an equivalent to 1000 Daltons and is used in biochemistry as a weight designation because biomolecules are generally quite large.
Ligation	The process of joining two separate parts as in creating a chemical bond between two atoms.
Macromolecular	A very large molecule, such as a polymer or protein, consisting of many smaller structural units linked together.

Millimolar	The concentration of a material in a solution. Millimolar is 1/1000 the concentration of a molar solution generally defined as the number of moles of substance in one liter of solution. A mole is defined as the number of elementary components such as atoms that is equivalent to 12 grams of carbon. A common historical convention has been to relate this number to Avagadro's constant which is 6.022×10^{23}.
Molecular Mass	Also termed molecular weight is a measure of the weight of a molecule based upon the sum of the individual weights of the constitutive atoms. Generally, this measurement is determined by comparison to the standard weight of 1/12 of the mass of the element carbon in similar manner to the measurement of a mole.
Nucleophile	A chemical compound or group that is attracted to nuclei and tends to donate or share electrons.
pH	The inverse logarithmic concentration of hydrogen ions in a solution. In general terms this is simply the measurement of a solutions acidic or basic character.
Polypeptide	An assembly or polymer of molecules that are composed of amino acids linked together by amide bonds. These polymers constitute the basic building blocks of proteins and enzymes.
Proteolysis	The hydrolysis or cleavage of amide bonds found in polypeptides to produce shorter polypeptides or individual amino acids.
Recombinant	DNA in which one or more segments or genes have been inserted, either naturally or by laboratory manipulation, from a different molecule or from another part of the same molecule, resulting in a new genetic combination.
Sequestration	The action of forming a chelate or other stable compound with an ion or atom or molecule so that it is no longer available for reactions.
Thermophile	An organism that thrives at a temperature of 50°C or higher.